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Enantioselective Hydroxylation of 4-Alkylphenols by Vanillyl Alcohol Oxidase

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Abstract: Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* catalyzes the enantioselective hydroxylation of 4-ethylphenol, 4-propylphenol, and 2-methoxy-4-propylphenol into 1-(4'-hydroxyphenyl)ethanol, 1-(4'-hydroxyphenyl)propanol, and 1-(4'-hydroxy-3'-methoxyphenyl)propanol, respectively, with an ee of 94% for the *R* enantiomer. The stereochemical outcome of the reactions was established by comparing the chiral GC retention times of the products to those of chiral alcohols obtained by the action of the lipases from *Candida antarctica* and *Pseudomonas cepacia*. Isotope labeling experiments revealed that the oxygen atom incorporated into the alcoholic products is derived from water. During the VAO-mediated conversion of 4-ethylphenol/4-propylphenol, 4-vinylphenol/4-propenylphenol are formed as side products. With 2-methoxy-4-propylphenol as a substrate, this competing side reaction is nearly abolished, resulting in less than 1% of the vinylic product, isoeugenol. The VAO-mediated conversion of 4-alkylphenols also results in small amounts of phenolic ketones indicative for a consecutive oxidation step. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* 59: 171–177, 1998.

Keywords: 4-alkylphenols; vanillyl alcohol oxidase; covalent flavoprotein; enantioselectivity; 4-vinylphenol

INTRODUCTION

The enantioselective oxidation of aromatic compounds for the production of chiral synthons and other fine chemicals by various kinds of biocatalysts has received much attention during the last years (Crosby, 1991; Stinson, 1995; Zaks and Dodds, 1995). Relatively little is known about the enzymatic enantioselective hydroxylation of prochiral alkylphenols. It has been well established that certain bacterial flavocytochromes catalyze the oxygen-independent asymmetric synthesis of 1-(4'-hydroxyphenyl)alkanols from 4-alkylphenols (McIntire et al., 1984; Bossert et al., 1989; Reeve et al., 1989), but their use in biotechnology applications is

limited by the need of artificial electron acceptors. Oxidation reactions of 4-alkylphenols by heme-dependent enzymes like cytochrome P450 and horseradish peroxidase have also been reported (Thompson et al., 1989, 1995). However, heme-based oxidations of 4-alkylphenols result in the formation of highly unstable *p*-quinone methides, leading to aspecific polymers as the main products.

Recently, we described a novel flavoprotein from *Penicillium simplicissimum* acting on aromatic compounds which uses oxygen as mild reoxidant and is relatively stable (van Berkel et al., 1994). This enzyme, referred to as vanillyl alcohol oxidase (VAO), is a homooctamer of 520 kDa with each subunit containing 8 α -(*N*³-histidyl)FAD as a covalently bound prosthetic group (de Jong et al., 1992). Besides oxidizing vanillyl alcohol to vanillin, VAO is also able to hydroxylate, deaminate, and demethylate a variety of phenolic compounds (Fraaije et al., 1995).

The reaction mechanism of flavoprotein-mediated hydroxylation of 4-alkylphenols has been studied to some extent. The hydroxylation of 4-ethylphenol by the bacterial flavocytochrome *p*-cresol methylhydroxylase (PCMH) from *Pseudomonas putida* has been proposed to involve the initial formation of an enzyme-bound *p*-quinone methide product intermediate (Hopper, 1976; McIntire and Bohmont, 1987). Addition of water then results in the formation of 1-(4'-hydroxyphenyl)ethanol (Hopper, 1978). A similar reaction mechanism has been proposed for the VAO-catalyzed conversion of 4-allylphenols and is supported by the observation that the competitive inhibitor isoeugenol binds in its deprotonated form (Fraaije et al., 1995). Formation of the *p*-quinone methide is followed by an enzyme-mediated addition of water to the electrophilic methide moiety. Recently, we obtained from rapid reaction studies the first spectral evidence for the formation of *p*-quinone methide intermediates in VAO-catalyzed reactions (Fraaije and van Berkel, 1997).

In this report we focus on the stereochemistry of the

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VAO-mediated hydroxylation of 4-alkylphenols. It is demonstrated that VAO is very enantioselective in the hydroxylation of 4-ethylphenol, 4-propylphenol, and 2-methoxy-4-propylphenol. Furthermore, as 4-vinylphenols are well-known flavour compounds (Edlin et al., 1995), attention was paid to the accumulation of these side products as well.

MATERIALS AND METHODS

GC analysis was performed on a Fisons 8160 gas chromatograph equipped with a FID ($T = 260^{\circ}\text{C}$). The chiral column used was a 30-m β -Dex fused silica capillary column (0.25 mm internal diameter; film thickness 0.25 μm), having permethylated β -cyclodextrin as the chiral stationary phase. H_2 was used as a carrier gas; flow = 0.7 mL min^{-1} . The initial temperature was 80°C . After sample injection, the temperature was raised $7^{\circ}\text{C min}^{-1}$ up to 150°C (130°C for (*R,S*)-1-(4'-hydroxy-3'-methoxyphenyl)propanol), which was held until all products were detected.

GC/MS experiments were performed on a Hewlett Packard (HP) 5890 gas chromatograph with a 30-m DB-17 column and a HP 5970 MSD. The initial temperature was 80°C . After injection, the temperature was raised $7^{\circ}\text{C min}^{-1}$ up to 240°C . Mass spectrum of 4-vinylphenol, m/z (relative abundance): 120 (M^+ , 100), 119 (24), 91 (49), 65 (23), 39 (20). Mass spectrum of 4-propenylphenol, m/z (relative abundance): 134 (M^+ , 100), 133 (85), 107 (37), 105 (48), 91 (22), 79 (21), 77 (38), 51 (25), 39 (29).

HPLC analysis was performed on a Spark Holland instrument with the use of a Gynkotek M480 pump and an Applied Biosystems 759A double beam UV/VIS detector. The column used was an S5-ODS 2 (250 mm \times 4.6 mm). The solvent used was methanol/water (1:1) at a flow rate of 0.9 mL min^{-1} . Aromatic compounds were detected at 276 nm. The integrator used was an HP 3395.

^1H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Samples were dissolved either in pyridine- d_5 or CDCl_3 (with TMS as internal standard). Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Elemental analysis was carried out on a Carlo Erba elemental analyser 1106.

Chemicals

4-Hydroxyacetophenone, imidazole, and vanillin were products of Janssen Chimica; *p*-cresol, 4-ethylphenol, 4-propylphenol, *tert*-butyldimethylsilyl chloride (TBDMSCl), 2-methoxy-4-propylphenol, and H_2^{18}O were obtained from Aldrich. Ethyl bromide was obtained from Merck. 4-Vinylphenol was purchased from Lancaster. Tetrabutylammonium fluoride (TBAF) (1 *M* in tetrahydrofuran) was from Acros. All solvents used were distilled before use.

(*R,S*)-1-(4'-Hydroxyphenyl)ethanol

To a stirred solution of 3.4 g (0.025 mol) of *p*-hydroxyacetophenone in 50 mL of methanol was added a solution of

sodium borohydride (0.57 g, 0.015 mol of NaBH_4 in 10 mL of 0.2 *M* NaOH) at a rate of 0.5 mL min^{-1} , with occasional cooling to keep the temperature at 18 – 25°C . The reaction was followed by TLC with chloroform/methanol (20:1) as eluent. When the reaction was complete, most of the methanol was removed by evaporation and the residue was diluted with 100 mL of water. The mixture was extracted with ether, after which the ether layer was washed with water and dried over magnesium sulphate. The ether was removed by evaporation, yielding a mixture of (*R,S*)-1-(4'-hydroxyphenyl)ethanol and residual 4-hydroxyacetophenone. 4-Hydroxyacetophenone was removed by washing with chloroform, yielding 300 mg of pure (*R,S*)-1-(4'-hydroxyphenyl)ethanol. Mp 130.6 – 131°C (mp 132 – 133°C for *R/S* mixture; McIntire et al., 1984). ^1H NMR (d_5 -pyridine) δ_{H} (ppm): 1.67 (d, 3H, $-\text{CH}_3$), 5.16 (q, 1H, CH), 7.23 and 7.57 (2 \times d, 4H, Ar-H). Mass spectrum, m/z (relative abundance): 138 (M^+ , 26), 123 (100), 95 (76), 77 (56), 65 (23), 43 (41), 39 (28). HRMS calcd for $\text{C}_8\text{H}_{10}\text{O}_2$ m/z 138.0681, found m/z 138.0681. Elemental anal. calcd for $\text{C}_8\text{H}_{10}\text{O}_2$: C, 69.54; H, 7.30. Found: C, 69.81; H, 7.42. These values are all in accordance with the values reported by Everhart and Craig (1991).

(*R,S*)-1-(4'-Hydroxyphenyl)propanol

To a stirred solution of 3.75 g (0.025 mol) of 4-hydroxypropiophenone in 50 mL of methanol was added a solution of sodium borohydride (0.57 g, 0.015 mol of NaBH_4 in 10 mL of 0.2 *M* NaOH) at a rate of 0.5 mL min^{-1} , with occasional cooling to keep the temperature at 18 – 25°C . The next steps in the synthesis were the same as for the synthesis of (*R,S*)-1-(4'-hydroxyphenyl)ethanol. In the final step, the product was purified by silica column chromatography yielding 1.2 g of pure (*R,S*)-1-(4'-hydroxyphenyl)propanol. ^1H NMR (CDCl_3) δ_{H} (ppm): 0.90 (t, 3H, CH_3), 1.73–1.80 (m, 2H, CH_2), 4.55 (t, 1H, CH), 6.77 and 7.14 (2 \times d, 4H, Ar-H). Mass spectrum, m/z (relative abundance): 152 (M^+ , 10), 123 (100), 95 (51), 77 (41), 39 (16).

(*R,S*)-1-(4'-Hydroxy-3'-methoxyphenyl)propanol (Fig. 1)

4-[(1-*tert*-Butyl-1,1-dimethylsilyl)oxy]-3-methoxybenzaldehyde (**2**)

To a stirred solution of 2 g (13.2 mmol) of 4-hydroxy-3-methoxybenzaldehyde (**1**) in 20 mL of DMF were added 1.79 g (26.4 mmol) of imidazole and 2.38 g (15.8 mmol) of TBDMSCl. The mixture was stirred at room temperature under a nitrogen atmosphere for 40 h and then poured into 80 mL of water. The mixture was extracted twice with 100 mL of petroleum ether (bp 40 – 60°C), which was subsequently washed with 100 mL of brine, dried over MgSO_4 , and evaporated, yielding 3.75 g of **2** as a yellow oil which was used for the Grignard reaction without further purification. ^1H NMR (CDCl_3) δ_{H} (ppm): 0.21 (s, 6H, Si- CH_3),

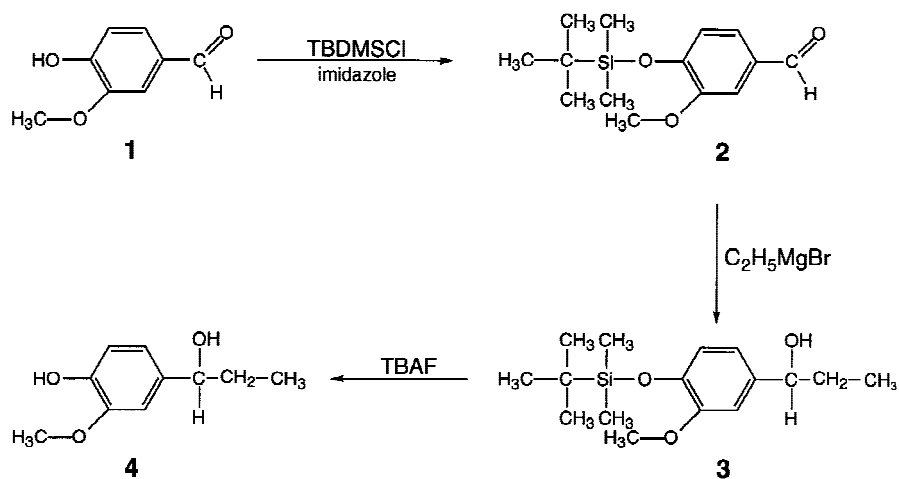


Figure 1. Scheme for the synthesis of 1-(4'-hydroxy-3'-methoxyphenyl)propanol.

1.03 (s, 9H, C₄H₉), 3.89 (s, 3H, OCH₃), 6.95–7.41 (m, 3H, Ar-H), 9.86 (s, 1H, CHO). Mass spectrum, *m/z* (relative intensity): 209 (M⁺ – C₄H₉, 81), 195 (18), 194 (100), 193 (44), 59 (23), 57 (17), 41 (29), 29 (31).

1-{4'-[(1-*tert*-Butyl-1,1-dimethylsilyl)oxy]-3'-methoxyphenyl}propanol (**3**)

In a three-necked flask equipped with a reflux condenser, dropping funnel, and a stirrer was placed 0.36 g of magnesium turnings. To this was added anhydrous ether (dried over sodium) until all the magnesium turnings were covered. The whole setup was under a nitrogen atmosphere. A small crystal of I₂ was added, after which a few drops of ethyl bromide were added until the reaction started. Hereafter, 1.64 g of ethyl bromide in 20 mL of anhydrous ether was dropped to the stirred solution as rapidly as the refluxing of the ether allowed. When all the ethyl bromide was added, the reaction was stirred for another hour.

A solution of 3.5 g of **2** in 20 mL of anhydrous ether was dropped to the stirred Grignard reagent. When all of **2** was added, the reaction mixture was heated (with gentle boiling) for another hour. The reaction mixture was poured into a solution of 10 g of NH₄Cl in 120 mL of crushed ice. The resulting mixture was extracted twice with 150 mL of ether and washed with 100 mL of brine. The ether was evaporated, yielding 3.03 g of **3** as a yellow oil which was used without further purification. ¹H NMR (CDCl₃) δ_H (ppm): 0.17 (s, 6H, Si-CH₃), 0.92 (t, 3H, CH₃), 1.02 (s, 9H, C₄H₉), 1.78–1.86 (m, 2H, CH₂), 3.89 (s, 3H, OCH₃), 4.55 (t, 1H, CH), 6.73–6.88 (m, 3H, Ar-H). Mass spectrum, *m/z* (relative intensity): 239 (M⁺ – C₄H₉, 61), 195 (100), 167 (22), 75 (18), 73 (30), 59 (21), 57 (25), 41 (21), 29 (31).

(*R,S*)-1-(4'-Hydroxy-3'-methoxyphenyl)propanol (**4**)

To a stirred solution of 2.0 g of **3** in 40 mL of dry DMF was added 16 mL of TBAF (1 M in tetrahydrofuran). The reaction mixture was stirred at room temperature until the reac-

tion was complete (90 min). The resulting green mixture was poured into 50 mL of water and extracted twice with 100 mL of ether. The ether was washed with brine, dried over Na₂SO₄, and evaporated. Purification of the reaction product by silica column chromatography (eluent: 5% MeOH in CHCl₃) gave 0.46 g (20%) of (*R,S*)-1-(4'-hydroxy-3'-methoxyphenyl)propanol. ¹H NMR (CDCl₃) δ_H (ppm): 0.91 (t, 3H, CH₃), 1.73–1.87 (m, 2H, CH₂), 3.89 (s, 3H, OCH₃), 4.55 (t, 1H, CH), 6.77–6.90 (m, 3H, Ar-H). Mass spectrum, *m/z* (relative intensity): 182 (M⁺, 23), 153 (92), 125 (38), 93 (100), 65 (52), 53 (16), 39 (19), 29 (33), 27 (28). HRMS calcd for C₁₀H₁₄O₃ *m/z* 182.0943, found *m/z* 182.0943.

Enzymes

Vanillyl alcohol oxidase (EC 1.1.3.7) was purified from *P. simplicissimum* (Oudem.) Thom. CBS 170.90 (ATCC 90172) as described by Fraaije et al. (1995). Lipase (EC 3.1.1.3) from *Pseudomonas cepacia* was a gift from Biocatalysts Ltd. (Pontypridd, UK). Lipase from *Candida rugosa* was purchased from Sigma, and lipase B from *Candida antarctica* was a generous gift from Boehringer (Mannheim, Germany).

Enzyme Kinetics

VAO activity was measured at pH 10.0 by spectrophotometrically recording the formation of aromatic product at 260 nm (to determine *K_m* values) and by following oxygen consumption using a Clark electrode (to determine *k_{cat}* values) (Fraaije et al., 1995).

Enzymatic Conversion of 4-Alkylphenols

The reaction mixture containing 1.0 mM 4-ethylphenol in 50 mM glycine/NaOH buffer, pH 10.0, and 0.05 U of VAO in 2 mL was incubated for 3 h at 30°C. Product samples

were prepared by extraction of the reaction mixture with 2×1.0 mL of ether. After evaporation, the products were dissolved in 10 μ L of methanol and analyzed using GC and GC/MS. For larger scale accumulation of the alcohol product formed after the enzymatic conversion of 4-ethylphenol, 15 mg of 4-ethylphenol and 100 μ g of VAO in 50 mL of 50 mM glycine/NaOH (pH 10.0) were incubated at 30°C. Samples of 0.5 mL were extracted with 1 mL of ether and analyzed by HPLC. When all 4-ethylphenol was consumed, another 15 mg of 4-ethylphenol was added. Further additions of 4-ethylphenol were made to a total of 100 mg. Isolation of the products was done essentially according to Reeve et al. (1990). The reaction mixture was extracted two times with 100 mL of ether, dried over Na_2SO_4 , and evaporated. The solid obtained was washed with 20 mL of light petroleum (bp 40–60°C) to remove 4-ethylphenol and 4-vinylphenol and with 20 mL of chloroform to remove 4-hydroxyacetophenone, yielding 33.2 mg (28%) of 1-(4'-hydroxyphenyl)ethanol. The conditions for the enzymatic conversion of 4-propylphenol and 2-methoxy-4-propylphenol were the same as for the oxidation of 4-ethylphenol. A large scale conversion was also performed for 2-methoxy-4-propylphenol. After extraction, the alcohol product was purified by column chromatography (eluent: 5% MeOH in CHCl_3), yielding 52 mg (45%) of 1-(4'-hydroxy-3'-methoxyphenyl)propanol as a yellow oil.

For ^{18}O incorporation experiments, 0.5-mL samples of 1.0 mM 4-ethylphenol were freeze-dried and resuspended in 0.5 mL of 33% w/w H_2^{18}O . After addition of vanillyl alcohol oxidase (100 μ g) the samples were incubated for 3 h at 25°C. Hereafter, the samples were extracted twice with 0.5 mL of ether, evaporated, and analyzed by GC/MS.

Establishment of the Absolute Configurations of Chiral Products

The absolute configuration of the hydroxyalkylphenols produced by VAO were determined using a chiral GC column in combination with the well-established stereoselectivity of the lipases from *P. cepacia* and *C. antarctica*, essentially according to the procedure of Reeve et al. (1990). A typical experiment is described below.

Racemic 1-(4'-hydroxyphenyl)ethanol was injected on a β -cyclodextrin chiral GC column. The enantiomers were well separated, giving peaks at 49.3 and 51.2 min. Subsequently, this alcohol was enzymatically esterified with vinyl acetate. Racemic 1-(4'-hydroxyphenyl)ethanol (40 mg) was dissolved in 2 mL of freshly distilled vinyl acetate, and 100 mg of lipase from *P. cepacia* was added. The mixture was incubated at 28°C. At regular time intervals, the lipase was removed by centrifugation and the supernatant was analyzed on the chiral column. During the first 50 h of the reaction, the peak at 49.3 min decreased whereas the area of the peak at 51.2 min remained unchanged. Earlier studies of *P. cepacia* lipase have revealed that it very selectively reacts with secondary alcohols having the 3D structure as shown below in Fig. 2 (Kazlauskas et al., 1991; Cygler et al.,

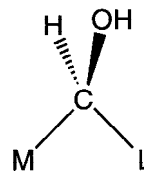


Figure 2. Stereochemical preference of *P. cepacia* lipase toward secondary alcohols. "L" represents a large substituent; "M" represents a medium-sized substituent.

1994). This means in our case that the lipase preferably reacts with the *R*-alcohol, leaving the *S*-alcohol unchanged. From these results, we could assign the *R*-structure to the peak at 49.3 min and the *S*-configuration to the peak at 51.2 min. Injection of the product obtained by VAO-mediated hydroxylation of 4-ethylphenol on the chiral GC column under the same conditions showed a large peak at 49.3 min and a very small one at 51.2 min, which leads to the conclusion that VAO produces *R*-1-(4'-hydroxyphenyl)ethanol with 94% ee.

An identical experiment with racemic 1-(4'-hydroxyphenyl)propanol and *P. cepacia* lipase gave peaks at 61.2 and 62.5 min; the peak at 61.2 min decreased during incubation with the lipase and vinyl acetate. The main product of the reaction of 4-propylphenol with VAO displayed a peak at 61.2 min, which means that VAO produces *R*-1-(4'-hydroxyphenyl)propanol (94% ee). Since both *P. cepacia* and *C. rugosa* lipase were unreactive toward 1-(4'-hydroxy-3'-methoxyphenyl)propanol, the lipase of *C. antarctica* was used. This enzyme is also very stereoselective toward secondary alcohols and has the same stereochemical preference as the other two lipases according to literature (Uppenberg et al., 1995). Indeed, in our hands *C. antarctica* lipase displayed the same stereochemical preference for 1-(4'-hydroxyphenyl)ethanol as *P. cepacia* lipase, i.e. the peak of the *R*-isomer decreased during incubation with the lipase and vinyl acetate. Racemic 1-(4'-hydroxy-3'-methoxyphenyl)propanol gave peaks at 139.7 and 144.6 min; *C. antarctica* lipase-mediated esterification in vinyl acetate resulted in a decrease of the peak at 139.7 min while the area of the peak at 144.6 min remained unchanged. Incubation of VAO with 2-methoxy-4-propylphenol gave a large peak at 139.7 min and a small one at 144.6 min, so VAO produces *R*-1-(4'-hydroxy-3'-methoxyphenyl)propanol (94% ee).

RESULTS

Steady-state kinetics were performed in order to determine the catalytic efficiency of VAO with 4-alkylphenols. The kinetic parameters for 4-ethylphenol, 4-propylphenol, and 2-methoxy-4-propylphenol (Table I) were in the same range as reported for other VAO substrates (Fraaije et al., 1995).

HPLC analysis showed that two major products are formed during the enzymatic oxidation of 4-ethylphenol. GC/MS and ^1H NMR analysis revealed that one of these major products is 1-(4'-hydroxyphenyl)ethanol (78%). The

Table I. Kinetic parameters for the conversion of 4-alkylphenols by vanillyl alcohol oxidase from *P. simplicissimum* (pH 10.0, 25°C).

Substrate		k_{cat} (s ⁻¹)	K_m (μM)
4-Ethylphenol		2.7	7
4-Propylphenol		3.9	3
2-Methoxy-4-propylphenol		4.8	<10

other major product was 4-vinylphenol (18%) according to GC/MS analysis. The vinylic product was not formed by autocatalysis as prolonged incubation of either 4-ethylphenol or 1-(4'-hydroxyphenyl)ethanol in the absence of VAO did not result in the formation of 4-vinylphenol. Conversion of 4-ethylphenol also resulted in some formation of 4-hydroxyacetophenone (4%) indicative of some subsequent VAO-mediated oxidation of the formed alcohol.

Similar results as with 4-ethylphenol were obtained for the VAO-catalyzed conversion of 4-propylphenol. This reaction resulted in the formation of 1-(4'-hydroxyphenyl)propanol (78%), 4-propenylphenol (18%), and 1-(4'-hydroxyphenyl)propanone (4%). HPLC, GC/MS, and ¹H NMR analysis revealed that 2-methoxy-4-propylphenol was mainly converted by VAO into 1-(4'-hydroxy-3'-methoxyphenyl)propanol. In contrast to the above-mentioned reactions, only minor amounts (<1%) of the unsaturated product, isoeugenol, and 1-(4'-hydroxy-3'-methoxyphenyl)propanone were formed in this case.

In order to identify the origin of the oxygen atom incorporated in the alcohol product, conversion of 4-ethylphenol by VAO was performed in H₂¹⁸O-enriched buffer. The mass spectrum of 1-(4'-hydroxyphenyl)ethanol, isolated from the reaction mixture enriched with H₂¹⁸O, resulted in an additional ion at m/z 140 (= $M^+ + 2$) (Fig. 3). This indicates that the incorporated oxygen atom is derived from water. The labeled alcohol represented 34% of the total amount of alcohol product, which is in good agreement with the 33% enrichment of H₂¹⁸O in the reaction mixture.

The absolute configuration of the chiral alcohols formed during the VAO-mediated conversion of 4-alkylphenols was determined using a chiral GC-column and lipase from *P. cepacia* and *C. antarctica*. These lipases specifically esterify the *R*-isomer of secondary alcohols (see Reeve et al., 1990, and Kazlauskas et al., 1991, for *P. cepacia* lipase and Uppenberg et al., 1995, for *C. antarctica* lipase). This procedure revealed that hydroxylation of 4-ethylphenol occurs enantioselectively, giving the *R*-isomer with an ee of 94%. Optical purity analysis of purified 1-(4'-hydroxyphenyl)ethanol ($[\alpha]_D^{20} = +40.1^\circ$) resulted in an ee of 82% (Everhart and Craig, 1991). ¹H NMR revealed that this ap-

parent lower ee resulted from the presence of some 4-hydroxyacetophenone. The positive optical rotation is in accordance with the optical rotation measured by Reeve et al. (1990) and Everhart and Craig (1991), confirming that (*R*)-1-(4'-hydroxyphenyl)ethanol is the main product formed from 4-ethylphenol. Furthermore, the melting point of the purified alcohol (148–151°C) again confirmed enantioselective hydroxylation (mp for one enantiomer, 157°C (Everhart and Craig, 1991); mp for *R/S* mixture, 130.6–131°C (see Materials and Methods)).

Stereochemical analysis of the 4-hydroxybenzylic alcohols formed from 4-propylphenol and 2-methoxy-4-propylphenol again revealed a 94% ee in favour of the *R*-isomer. Lipase treatment showed that VAO predominantly hydroxylates 4-propylphenol to the *R*-enantiomer of 1-(4'-hydroxyphenyl)propanol.

DISCUSSION

This paper reports on the asymmetric synthesis of optically active short-chain 1-(4'-hydroxyphenyl)alkanols by the covalent flavoprotein vanillyl-alcohol oxidase from *P. simplicissimum*. Hydroxylation of 4-ethylphenol resulted in the enantioselective formation of (*R*)-1-(4'-hydroxyphenyl)ethanol with an ee of 94%. For the hydroxylation of 4-propylphenol and 2-methoxy-4-propylphenol also an ee of 94% for the *R*-isomer was found. Previous studies have shown that the bacterial flavocytochromes PCMH (McIntire and Bohmont, 1987) and 4-ethylphenol methylenehydroxylase (EPMH) (Reeve et al., 1990), both isolated from *P. putida* strains, also enantioselectively hydroxylate 4-ethylphenol. In case of PCMH, (*S*)-1-(4'-hydroxyphenyl)ethanol was formed with an ee of 31.2% when phenazine methosulphate was used as electron acceptor (McIntire et al., 1984), whereas an ee of 94% for the *S*-isomer was found with cytochrome *c* as electron acceptor (McIntire and Bohmont, 1987). On the other hand, more

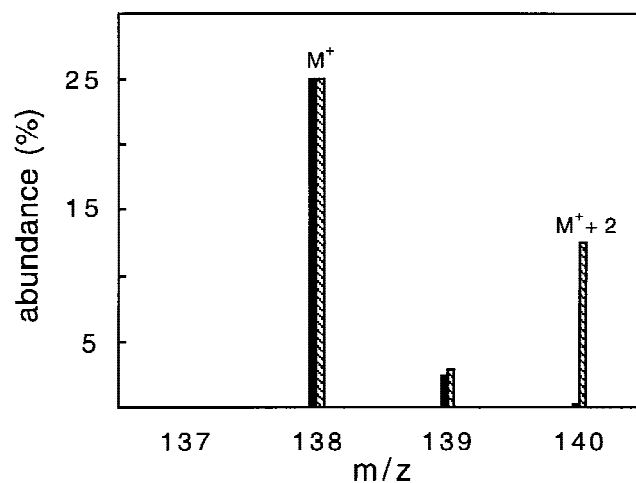


Figure 3. Mass spectra (M^+ – peak) of 1-(4'-hydroxyphenyl)ethanol obtained after incubation of 4-ethylphenol with VAO in the absence (solid bars) or presence (striped bars) of H₂¹⁸O.

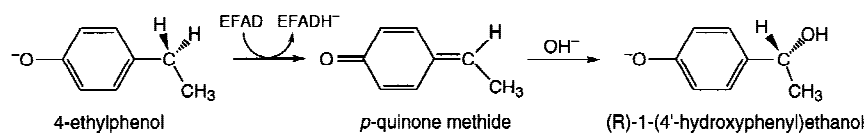


Figure 4. Proposed mechanism for the hydroxylation of 4-ethylphenol by vanillyl alcohol oxidase (EFAD, oxidized enzyme; EFADH⁻, reduced enzyme).

similar with VAO, EPMH catalyzes the hydroxylation of 4-ethylphenol into (*R*)-1-(4'-hydroxyphenyl)ethanol with an ee of 98% (Reeve et al., 1990). However, no data have been reported for the enantioselectivity of the bacterial flavocytochromes with 4-propylphenols.

Using H₂¹⁸O it was found that in the VAO-catalyzed reactions, the oxygen atom introduced in the alcohol product originates from water. This is in line with the reaction mechanism postulated for the VAO-mediated conversion of 4-(methoxymethyl)phenol (Fraaije and van Berkel, 1997), involving the initial formation of a *p*-quinone methide product intermediate. As the addition of water to the *p*-quinone methide is enzyme-mediated (Fraaije and van Berkel, 1997), it is apparent that the reaction with prochiral 4-alkylphenols results in optical active products (Fig. 4).

During the VAO-catalyzed conversion of 4-alkylphenols, 4-vinylc phenols are formed as side products (Fig. 5). McIntire and Bohmont (1987) have suggested that the formation of these unsaturated compounds might result from the rearrangement of the *p*-quinone methide intermediate. Interestingly, almost no isoeugenol was formed in the VAO-mediated conversion of 2-methoxy-4-propylphenol. This shows that introduction of substituents in the substrate aromatic ring may influence the absolute yield of chiral product formed. Rapid reaction studies have indicated that the electrophilic *p*-quinone methide product intermediates become stabilized in the active site of the reduced enzyme to a different extent, depending on the substrate, and that nucleophilic attack by water to these intermediates only occurs after flavin reoxidation (Fraaije et al., 1997). This suggests that the differences in the extent of formation of unsaturated products from 4-propylphenol and 2-methoxy-4-propylphenol result from subtle changes in susceptibility of the corresponding *p*-quinone methide intermediates toward rearrangement in the active site of the reduced enzyme.

VAO oxidizes 4-hydroxybenzylalcohols and catecholamines to the corresponding aldehydes or ketones (Fraaije et al., 1995). However, during the conversion of 4-alkylphenols, VAO produces only limited amounts of 4-hydroxyacetophenones. The inefficient formation of these compounds may be caused by the relatively weak binding of the 1-(4'-hydroxyphenyl)alkanols and the fact that the vinylic side products act as strong competitive inhibitors (Fraaije et al., 1995). A similar inhibition by 4-vinylphenol was reported in case of PCMH (McIntire and Bohmont, 1987). Because nearly no isoeugenol is formed from 2-methoxy-4-propylphenol, another likely explanation for the low yield of 4-hydroxyacetophenones is that VAO may be enantioselective in oxidizing preferably the *S*-isomers of 1-(4'-hydroxyphenyl)alkanols, while the *R*-isomers are predominantly formed.

Recently, the crystal structure of VAO has been determined in the native state and in complex with several inhibitors (Mattevi et al., 1997). These studies clearly established that the structure of the VAO subunit closely resembles that of the flavoprotein subunit of PCMH (Mathews et al., 1991). The crystal structure has provided a rationale for the poor reactivity of VAO toward 4-methylphenol (Fraaije et al., 1997), the physiological substrate of PCMH. Upon binding of 4-methylphenol, a covalent adduct is formed between the substrate and the reduced flavin which is rather stable under aerobic conditions (Mattevi et al., 1997). Steric constraints imposed by the shape of the active-site cavity most likely prevent such an adduct formation with more bulkier 4-alkylphenols, which is consistent with the present results.

In summary, this study strongly supports an earlier conclusion (Fraaije et al., 1995) that the reaction mechanism of VAO is similar to PCMH and EPMH. However, several marked differences exist between the catalytic properties of

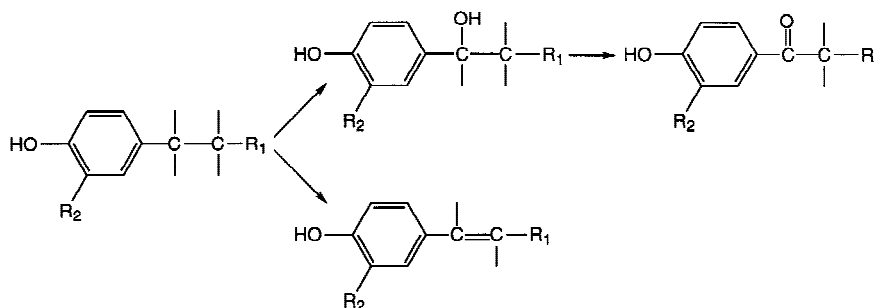


Figure 5. Reaction pathway for the conversion of short-chain 4-alkylphenols catalyzed by VAO (4-ethylphenol, R₁, R₂ = H; 4-propylphenol, R₁ = CH₃, R₂ = H; 2-methoxy-4-propylphenol, R₁ = CH₃, R₂ = OCH₃).

the bacterial flavocytochromes and VAO. These differences include the substrate specificity and the nature of the electron acceptor involved in flavin reoxidation. In view of potential biotechnological applications, it should be stressed that, opposite to the flavocytochromes, VAO does not need an artificial electron acceptor for flavin reoxidation but uses dioxygen instead. Therefore, like glucose oxidase (Wilson and Turner, 1992) and D-amino acid oxidase (Butò et al., 1994), VAO can be classed among an emerging group of flavoprotein oxidases that catalyze transformations of industrial relevance.

NOMENCLATURE

coniferyl alcohol	4-hydroxy-3-methoxycinnamyl alcohol
ee	enantiomeric excess
EPMH	4-ethylphenol methylhydroxylase
eugenol	4-allyl-2-methoxyphenol
isoeugenol	1,2-(methylenedioxy)-4-propenylbenzene
k_{cat}	turnover rate
K_m	Michaelis–Menten constant
PCMH	<i>p</i> -cresol methylhydroxylase
<i>p</i> -cresol	4-methylphenol
TBAF	tetrabutylammonium fluoride
TBDMSCl	<i>tert</i> -butyldimethylsilyl chloride
TLC	thin-layer chromatography
U	unit of enzyme activity
vanillin	4-hydroxy-3-methoxybenzaldehyde
vanillyl alcohol	4-hydroxy-3-methoxybenzyl alcohol
VAO	vanillyl alcohol oxidase

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